Substitution of the cysteine 438 residue in the cytoplasmic tail of the glucagon-like peptide-1 receptor alters signal transduction activity

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Abstract

Several G-protein-coupled receptors contain cysteine residues in the C-terminal tail that may modulate receptor function. In this work we analysed the substitution of Cys⁴³⁸ by alanine in the glucagon-like peptide-1 (GLP-1) receptor (GLPR), which led to a threefold decrease in cAMP production, although endocytosis and cellular redistribution of GLP-1 receptor agonist-induced processes were unaffected. Additionally, cysteine residues in the C-terminal tail of several G-protein-coupled receptors were found to act as substrates for palmitoylation, which might modify the access of protein kinases to this region. His-tagged GLP-1 receptors incorporated ³H-palmitate. Nevertheless, substitution of Cys⁴³⁸ prevented the incorporation of palmitate. Accordingly, we also investigated

the effect of substitution of the consensus sequence by protein kinase C (PKC) Ser^{431/432} in both wild-type and Ala⁴³⁸ GLP-1 receptors. Substitution of Ser^{431/432} by alanine did not modify the ability of wild-type receptors to stimulate adenylate cyclase or endocytosis and recycling processes. By contrast, the substitution of Ser^{431/432} by alanine in the receptor containing Ala⁴³⁸ increased the ability to stimulate adenylate cyclase. All types of receptors were mainly internalised through coated pits. Thus, cysteine 438 in the cytoplasmic tail of the GLP-1 receptor would regulate its interaction with G-proteins and the stimulation of adenylate cyclase. Palmitoylation of this residue might control the access of PKC to Ser^{431/432}.

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Introduction

Glucagon-like peptide-1(7–36)amide (GLP-1) is an intestinal peptide synthesised in L-cells. It is also produced in the brain (Jin *et al.* 1988, Kreymann *et al.* 1989), where it exerts some effects on neurotransmitter release from selected brain nuclei (Mora *et al.* 1992, Calvo *et al.* 1995) and produces an inhibitory effect on food and water intake (Navarro *et al.* 1996, Tang-Christensen 1996, Turton *et al.* 1996). Subcutaneous administration of GLP-1(7–36)amide also elicits a reduction in food and water intake (Rodríguez de Fonseca *et al.* 2000). Similar results were found in human subjects when the peptide was administered peripherally (Gutzwiller *et al.* 1999). Administered subcutaneously, the peptide could be transported into the brain through the choroid plexus, which has a high density of GLP-1 receptors (GLPR) (Alvarez *et al.* 1996), or by blood–brain barrier-free organs, such as the subfornical organ and the area postrema (Orskov *et al.* 1996). It has also been suggested that this peptide acts as a neurotrophic factor. GLP-1 receptors are expressed at high levels in glial cells after mechanical injury (Chowen *et al.* 1999) and it

has recently been shown that GLP-1(7–36)amide can promote the proliferation and differentiation of PC12 cells (Perry *et al.* 2002).

GLP-1(7–36)amide also has a potent effect on glucose-dependent insulin secretion (Kreymann *et al.* 1987, Weir *et al.* 1989), and it also increases arterial blood pressure, heart rate (Barragán *et al.* 1994, 1996), and lung surfactant synthesis (Benito *et al.* 1998, Vara *et al.* 2001).

The effects of GLP-1(7–36)amide are mediated through a specific G-protein-coupled receptor (Thorens 1992, Dillon *et al.* 1993, Wheeler *et al.* 1993, Wei & Mojsov 1995, Alvarez *et al.* 1996) and its signalling on target cells is dependent on activation of the adenylate cyclase pathway (Thorens 1992, Wheeler *et al.* 1993). In this context, the third intracellular cytoplasmic loop is involved in coupling to G proteins and activation of the adenylate cyclase system (Takhar *et al.* 1996). Desensitisation of the GLP-1 receptor has been correlated with C-terminal tail phosphorylation. Homologous desensitisation produced by GLP-1(7–36)amide induces the phosphorylation of three serine doublets located at positions 441/442, 444/445 and 451/452 and decreases maximum cAMP production by

20% (Widmann *et al.* 1997). Heterologous desensitisation of the GLP-1 receptor by protein kinase C (PKC) is correlated with the phosphorylation of four serine doublets located at positions 431/432, 441/442, 444/445 and 451/452. Activation of PKC by phorbol esters also decreases maximum cAMP production by 30% (Widmann *et al.* 1996a,b), and the levels of phosphorylation induced by GLP-1 and phorbol esters are additive (Widmann *et al.* 1996b). Phosphorylation of the three serine doublets, Ser^{441/442}, Ser^{444/445} and Ser^{451/452}, at the cytoplasmic tail after agonist binding is related to receptor endocytosis (Widmann *et al.* 1997). Agonist-promoted phosphorylation of the G-protein-coupled receptors increases the affinity of β -arrestins and these act as a clathrin adaptor protein in the internalisation process (Goodman *et al.* 1996).

Most G-protein-coupled receptors have one or two cysteine residues in the carboxyl-terminal cytoplasmic tail, and palmitoylation of these cysteines has been reported for several members of this family of receptors (Bouvier *et al.* 1995a,b, Morello & Bouvier 1996). The palmitate tail anchors the cytoplasmic tail of the receptor into the plasma membrane to create a fourth intracellular loop, allowing a reversible and dynamic regulation (Bouvier *et al.* 1995a).

The aim of this study was to investigate whether cysteine 438 is a site of acylation and the function of Cys⁴³⁸ in signal transduction through GLP-1 receptors.

Materials and Methods

Mutagenesis of the GLP-1 receptor

A HindIII-XbaI fragment of the rat GLP-1 receptor cDNA (Thorens 1992) was subcloned into PUC 18. Site-directed mutagenesis of cysteine 438 was accomplished using the Transformer Site-directed Mutagenesis kit from Clontech (Palo Alto, CA, USA). Mutagenic primers were prepared: 5' CCCCTTAAGGCTCCCAC CAGC 3' to change Cys⁴³⁸ to Ala and to create a BfrI restriction site, and 5' CCCCTTAAGTGTCCCACC AGC 3' to create only a BfrI restriction site at nucleotide 1322. A selection primer, 5' GTGTATCTTATGCC AAGTACG 3', to eliminate a unique NdeI restriction site in the vector was also used. Clones BfrI/Ala⁴³⁸ and BfrI were selected and mutations were confirmed by sequencing using an automated LKB ALF DNA sequencer (Amersham Pharmacia Biotech, Barcelona, Spain). HindIII-XbaI mutant cDNA was then cloned into the expression vector to create pcDNA3-GLPR A⁴³⁸ and pcDNA3 GLPR BfrI.

For substitution of amino acids Ser^{431/432} by alanine, PCR site-directed mutagenesis was used, following standard procedures. The following primers were used: two flanking primers -GLPR-1 oligonucleotide, corresponding to nucleotides 969–993, and GLPR-2 oligonucleotide, corresponding to nucleotides 1384–1411 (Thorens 1992);

the specific mutation primers GLPR-3: 5'CAGAGGGA CGCCGCCATGAAACCCTC3' and GLPR-4: 5'GG TTTCATGGCGGCGTCCCCTCTGGATG were employed to mutate Ser^{431/432} into alanine residues. Mutation identity was verified by sequence analysis. The fragments containing the mutated gene were subcloned into the SacI/BfrI restriction sites of pcDNA3-GLPR BfrI and pcDNA3-GLPR A⁴³⁸ to create pcDNA3-GLPR A^{431/432} and pcDNA3-GLPR A^{431/432/438}.

N-terminal epitope-tagged GLP-1 receptors were constructed by inserting a FLAG epitope (DYKDDDDK) after the initial methionine using a PCR strategy. A HindIII-AlwNI fragment was replaced in wild-type and mutant A⁴³⁸ and designated pcDNA 3 tag-GLPR and pcDNA 3 tag-GLPR A⁴³⁸. Also, 6 × His-tagged GLP-1 receptors were constructed. BamHI-XbaI wild-type and mutant cDNAs were subcloned into pcDNA 3.1/His and designated pcDNA3-His GLPR and pcDNA3-His GLPR A⁴³⁸. Wild-type and mutant GLP-1 receptors were expressed in CHO cells by transfection using the calcium phosphate method. Transfected cells were selected by incubation with neomycin (geneticin, GIBCO). Stable colonies were isolated using cloning rings.

Binding of ¹²⁵I-GLP-1(7–36)amide to cell surface receptors

GLP-1(7–36)amide was radio-labelled with ¹²⁵I by the chloramine T method and mono ¹²⁵I-GLP-1(7–36)amide was isolated by reverse-phase HPLC. Specific activity was between 3500–4000 d.p.m./fmol. CHO cells grown in 24-well plates (2 × 10⁵/well) were washed with serum-free medium and incubated for 30 min at 37 °C in KRH (118 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM HEPES, pH 7.4) with 0.5% bovine serum albumin (BSA). Then, cells were chilled on ice, and ¹²⁵I-GLP-1(7–36)amide (0.1 nM) and unlabelled GLP-1(7–36)amide (0.0–500 nM) were added for at least 16 h at 4 °C. Following this, cells were washed three times with cold medium and solubilised with 1 M NaOH. Non-specific binding of ¹²⁵I-GLP-1(7–36)amide was estimated in the presence of 500 nM unlabelled ligand and this value was subtracted from the total binding values.

cAMP production assays

cAMP levels were measured using the cAMP Biotrack enzyme immunoassay system (Amersham Biosciences, UK) following the manufacturer's instructions. Briefly, untransfected CHO cells and cells expressing wild-type and mutant GLP-1 receptors were grown in 12-well plates in F-12 medium supplemented with 5% FBS. CHO cells were washed and incubated in F-12 medium containing 0.1% FBS overnight. Cells were then washed with HBS medium (130 mM NaCl, 0.9 mM NaH₂PO₄, 0.8 mM MgSO₄, 5.4 mM KCl, 1.8 mM CaCl₂, 20 mM HEPES, pH 7.4, plus 25 mM glucose) and incubated in HBS

containing 1 mM IBMX and 0.1% BSA in the presence or absence of different concentrations of GLP-1(7–36)amide (0.001–100 nM) for 8 min. Then, cells were washed with HBS, after which lysis buffer was added and immediately processed in the immunoassay. In parallel experiments, binding of ¹²⁵I-GLP-1(7–36)amide to cell surface receptors was determined at 4 °C. The agonist stimulated cAMP production was normalised by ¹²⁵I-GLP-1(7–36)amide bound to cell surface receptors.

Internalisation of the GLP-1 receptor

The internalisation rate was measured as previously described (Alvarez *et al.* 1990). Briefly, CHO cells were incubated in KRH (118 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM HEPES, pH 7.4) with 0.5% BSA at 37 °C for 30 min. Then, cells were incubated with 0.1 nM ¹²⁵I-GLP-1(7–36)amide, and ¹²⁵I-GLP-1(7–36)amide binding was determined at different times. Intracellular ¹²⁵I-GLP-1(7–36)amide was measured as cell-associated radioactivity resistant to acid wash by incubation of the cells for 3 min at 4 °C with 50 mM NaCl, 150 mM glycine, pH 3.0. Cell surface-bound ¹²⁵I-GLP-1(7–36)amide was calculated by subtraction of the intracellularly bound ¹²⁵I-GLP-1(7–36)amide from the total binding.

Effect of metabolic inhibitors and hypertonic medium on the internalisation process

For metabolic depletion, CHO cells were washed twice with glucose-free HBS (140 mM NaCl, 20 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM KCl with 0.5% BSA) and incubated in the same buffer containing 20 mM 2-deoxyglucose and 10 mM sodium azide. Hypertonic treatment was performed as previously described (Heuser & Anderson 1989). Briefly, cells were washed twice with HBS containing 0.45 M sucrose and incubated in the same medium for 30 min at 37 °C. Then, 0.1 nM ¹²⁵I-GLP-1(7–36)amide was added and after 6 min incubation cell-associated radioactivity and acid wash-resistant radioactivity were measured as previously described.

Redistribution of cell surface GLP-1 receptors

CHO cells were seeded in 24-well plates and incubated with 10 nM GLP-1(7–36)amide for different times (3, 6, 10, 15, 30 and 60 min). Then, the cells were washed at 0 °C and subsequently incubated for 3 min at 0 °C with 50 mM NaCl, 150 mM glycine, pH 3.0, to remove ligand bound to the cell surface, and washed with KRH containing 0.5% BSA. Cell surface receptors were then measured as described above.

Immunoprecipitation of tag-GLPR receptors

CHO cells expressing tag-GLPR wild-type or mutant receptors were seeded in 60 cm² dishes for metabolic

labelling. The cells were transferred into culture media: (1) methionine-free modified Eagle's medium containing 150 µCi/ml ³⁵S-methionine (Amersham Pharmacia Biotech) and (2) F-12 medium containing 1% fetal bovine serum (FBS) (GIBCO, Life Technologies, Paisley, UK) and 200 µCi/ml ³H-palmitate (Dupont-NEN, Boston, MA, USA). The cells were then washed and solubilised in PBS containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 5 mM Na₄P₂O₇ and 1 mM Na₃VO₄. The lysates were centrifuged at 10 000 g for 30 min and the supernatants were incubated with agarose-Streptavidin at room temperature for 30 min. After centrifugation, the supernatants were incubated for 60 min at room temperature with monoclonal anti-FLAG epitope M2-biotin conjugate antibody (SIGMA, Madrid, Spain) prebound to 50 µl agarose-Streptavidin or monoclonal anti-FLAG epitope M2-agarose (SIGMA). The immunoprecipitates were washed and resolved by SDS-PAGE and fluorography.

For purification of His-tagged GLP-1 receptors, crude membrane fractions of CHO cells expressing GLP-1 receptors were loaded into His micro spin columns (Amersham Biosciences Europe, Barcelona, Spain) previously incubated in the presence or absence of an excess of polyHis (30 µg/ml). Then, the columns were washed twice with phosphate/NaCl solution containing 20 mM imidazole and twice with phosphate/NaCl 60 mM imidazole and finally eluted with phosphate/NaCl solution containing 400 mM imidazole. Western blot analyses with anti-His antibody (Amersham Biosciences Europe) were performed following the manufacturer's instructions.

CHO cells expressing His-wild-type or mutant GLP-1 receptors were also labelled with ³⁵S-methionine or ³H-palmitate as described above. His-tagged GLP-1 receptors were purified using His micro spin columns and samples were resolved by SDS-PAGE and fluorography.

Data analysis

Data from competition experiments were analysed by non-linear regression with the GraphPad Prism computer program (GraphPad Software Inc., San Diego, CA, USA). Dissociation constants (K_d) and concentrations of receptor sites (B_{max}) were calculated from IC₅₀ values using the method of Cheng and Prusoff (1973). Data are expressed as means ± S.E.M. Statistical comparisons were performed using the Student's *t*-test and Newman-Keuls test with at least three separate experiments performed in duplicate (**P* < 0.05 and ***P* < 0.001).

Results

Effect of the mutation in Cys⁴³⁸ on GLP-1 receptor binding

To analyse the role of Cys⁴³⁸ located in the C-terminal domain of the GLP-1 receptor (Fig. 1), we constructed a

PRIMARY STRUCTURE

Wild-type	408	416	431/432	441/442	444/445	451/452	463
	EVQMEFRKSWERWRLERLNIQRDSSMKPLKCPTSSVSSGATVGSSVYAATCQNSCS						
GLPR A ⁴³⁸	408	416	431/432	441/442	444/445	451/452	463
	EVQMEFRKSWERWRLERLNIQRDSSMKPLK A PTSSVSSGATVGSSVYAATCQNSCS						
GLPR A ^{431/432}	408	416	431/432	441/442	444/445	451/452	463
	EVQMEFRKSWERWRLERLNIQRD A AMKPLKCPTSSVSSGATVGSSVYAATCQNSCS						
GLPR A ^{431/432/438}	408	416	431/432	441/442	444/445	451/452	463
	EVQMEFRKSWERWRLERLNIQRD A AMKPLK A PTSSVSSGATVGSSVYAATCQNSCS						

Figure 1 Amino acid sequence of the C-terminal tail of the wild-type and GLP-1 receptor mutants. The 56 C-terminal amino acids of the rat wild-type and mutant GLP-1 receptor are shown. Italicised letters represent substituted or mutated residues.

series of mutant receptors in which Cys⁴³⁸ or Ser^{431,432} were substituted by alanine (Fig. 1). CHO cells expressing wild-type or mutant receptors (GLPR A⁴³⁸, GLPR A^{431/432} and GLPR A^{431/432/438}) were used to determine the binding properties of ¹²⁵I-GLP-1(7–36)amide to all receptors. The competition binding curves are shown in Fig. 2. The K_d and B_{max} calculated from the IC₅₀ values are shown in Table 1. The competition binding data of wild-type and mutant receptors fit a single-site model best.

Figure 2 Analysis of ¹²⁵I-GLP-1(7–36)amide binding to wild-type and mutant GLP-1 receptors. CHO cells expressing wild-type, GLPR A⁴³⁸, GLPR A^{431/432} and GLPR A^{431/432/438} GLP-1 receptors were incubated with 0.1 nM ¹²⁵I-GLP-1(7–36)amide and increasing concentrations of the unlabelled peptide. The competition binding curves are shown. Data are means ± S.E.M. (n=3–6) and are expressed as a percentage of maximal specific binding.

Activation of adenylate cyclase

The binding of GLP-1(7–36)amide to its receptor produces activation of the adenylate cyclase pathway. Accordingly, we tested the effect of this peptide on the production of cAMP by cells transfected with the wild-type and mutant GLP-1 receptors. As shown in Fig. 3, GLP-1(7–36)amide stimulated CHO cells expressing wild-type receptors in a dose-dependent manner; the EC₅₀ of GLP-1(7–36) amide was 0.09 ± 0.03 nM, maximum stimulation (R_{max}) being observed at 10 nM of the agonist (Table 1). CHO cells expressing mutant GLPR A⁴³⁸ receptors showed a similar EC₅₀ 0.05 ± 0.016 nM value calculated from the dose–response curve (Fig. 3). However, the mutant receptor revealed a 60–70% decreased efficacy as compared with wild-type receptors (Table 1). Similar results were obtained using a pool of clones expressing the wild-type and mutant GLP-1 receptors.

Post-translational modifications of the GLP-1 receptor

To investigate post-translational modifications of the GLP-1 receptor by acylation, CHO cells expressing tag-wild-type GLP-1 receptors were labelled with ³⁵S-methionine and ³H-palmitic acid. The immunoprecipitates were analysed by non-reducing polyacrylamide gel electrophoresis and fluorography. The epitope-tagged GLP-1 receptors were not immunoprecipitated by anti-FLAG M2 monoclonal antibody or anti-FLAG M2 affinity gel, and hence we could not detect tag-GLP-1 receptors in cells labelled with ³⁵S-methionine. We therefore decided to use 6 × His-tagged GLP-1 receptors. His-GLP-1 receptors were isolated using His micro spin columns. Western blot analysis using an anti-His antibody (Fig. 4) revealed the presence of a 65 kDa band in CHO cells expressing His-tagged wild-type and mutant Ala⁴³⁸

Table 1 GLP-1(7-36)amide binding parameters and adenylyl cyclase stimulation for wild-type and mutant GLP-1 receptors expressed in CHO cells. The GLP-1(7-36)amide binding parameters were determined by competitive binding assays using CHO cells stably transfected with wild-type and mutant GLP-1 receptors. The data were analysed by non-linear regression and the dissociation constant (K_d) and the concentration of receptor sites (B_{max}) of these receptors were calculated from IC_{50} values as described in Materials and Methods. The data of adenylyl cyclase activity were analysed using non-linear least squares regression. The agonist potency EC_{50} and maximal GLP-1(7-36)amide-stimulated adenylyl cyclase activity (R_{max}) are shown. The data represent the mean $\pm$ S.E.M. of three to six independent experiments

Receptor	¹²⁵ I-GLP-1(7-36)amide binding parameters		Adenylyl cyclase stimulation	
	K_d (nM)	B_{max} (fmol/ 2×10^5 cells)	EC_{50} (nM)	R_{max} (pmol/ 2×10^5 cells)
Wild-type	1.46 ± 0.08	12 ± 1.65	0.09 ± 0.03	7.4 ± 1.5
GLPR A ⁴³⁸	0.905 ± 0.15	42.22 ± 6.41	0.05 ± 0.016	2.6 ± 0.6
GLPR A ^{431/432}	0.515 ± 0.03	30.05 ± 2.56	0.06 ± 0.02	6.6 ± 0.14
GLPR A ^{431/432/438}	0.44 ± 0.22	1.12 ± 0.4	0.44 ± 0.22	28.5 ± 5.7

GLP-1 receptors. This band disappeared when the columns were previously saturated with polyHis. CHO cells expressing His-tagged wild-type and mutant GLP-1 receptors were metabolically labelled with ³⁵S-methionine and ³H-palmitate. His-GLP-1 receptors were purified with His micro spin columns and subjected to SDS-PAGE and fluorography. As shown in Fig. 4, when the cells were labelled with ³⁵S-methionine a 65 kDa band was detected in cells transfected with wild-type and mutant GLPR A⁴³⁸ receptors. However, when the cells were labelled with ³H-palmitate, the incorporation of ³H-palmitate was only

Figure 3 Effect of GLP-1(7-36)amide on cAMP production by CHO cells transfected with wild-type and GLPR A⁴³⁸, GLPR A^{431/432} and GLPR A^{431/432/438} GLP-1 receptors. CHO cells expressing wild-type and mutant GLP-1 receptors were stimulated with different concentrations (0.001–100 nM) of GLP-1(7-36)amide for 8 min and cAMP production was determined by enzyme immunoassay and normalised by ¹²⁵I-GLP-1(7-36)amide bound to cell surface receptors. The data presented are means $\pm$ S.E.M. of three to six separate experiments performed in duplicate. * $P < 0.05$, ** $P < 0.001$, compared with cells expressing wild-type receptors (Neuman-Keuls test).

observed in cells expressing wild-type receptors. No ³H-palmitate-labelled band was detected in samples purified from CHO-K1 and mutant GLPR A⁴³⁸ receptor-expressing cell lines, although the expression levels of the wild-type and mutant GLPR A⁴³⁸ receptors were similar in this experiment, as indicated by the ³⁵S-labelled purification. These data are consistent with the hypothesis that Cys⁴³⁸ is a site of GLP-1 receptor palmitoylation.

The presence of cysteine residues in many G-protein-coupled receptors allows post-translational modification by palmitoylation. It has also been demonstrated that the palmitic acid tail can attach the cytoplasmic domain of the receptor to the cell membrane, which should change accessibility to the phosphorylation sites closest to Cys⁴³⁸ (Ser^{431,432} and Ser^{441,442}). In other experiments we used a receptor lacking Ser^{431,432}, the consensus phosphorylation site for PKC (GLPR A^{431/432/438}), but keeping the three serine doublets 441/442, 444/445 and 451/452, identified as the sites of homologous phosphorylation in response to agonist binding. To check the specific effect of substitution of Ser^{431,432} by alanine a mutant GLPR A^{431/432} was also used (Fig. 1).

Effect of substitution of Ser^{431/432} by alanine on adenylyl cyclase activation

Substitution of Ser^{431/432} by alanine in the wild-type receptor did not modify the potency and efficacy of such receptors in stimulating cAMP production (Fig. 3, Table 1). By contrast, in cells expressing GLPR A^{431/432/438} the EC_{50} value of GLP-1(7-36)amide was similar to that of the wild-type, but the efficacy was three- to fourfold increased relative to the wild-type (Table 1).

Effect of the Cys⁴³⁸ mutation and the substitution of Ser^{431/432} by alanine on the rate of GLP-1 receptor endocytosis

Figure 5A shows that the substitution of Cys⁴³⁸ or Ser^{431/432} by alanine in the GLP-1 receptor did not

Figure 4 Immunological detection of His-GLP-1 receptors and fluorography. (A) Eluates of the His micro spin column previously incubated with (+) or without (–) excess polyHis were processed by SDS-PAGE and Western blot. An immunoblot using an anti-His antibody is shown. Arrows indicate the positions of immunoreactive proteins. (B) CHO cells expressing His-GLPR and His-GLPR A⁴³⁸ receptors were labelled with ³⁵S-methionine and ³H-palmitate. His-GLP-1 receptors were purified with His micro spin columns and resolved by SDS-PAGE and fluorography. The figure presents an autoradiograph of the dried gel.

modify the rate of its internalisation. The first-order endocytic rates measured for wild-type, GLPR A⁴³⁸, GLPR A^{431/432} and GLPR A^{431/432/438} were $0.198 \pm 0.005 \text{ min}^{-1}$, $0.217 \pm 0.006 \text{ min}^{-1}$, $0.18 \pm 0.014 \text{ min}^{-1}$ and $0.22 \pm 0.015 \text{ min}^{-1}$ respectively. The rate of endocytosis of GLP-1 receptors was also similar in a pool of clones expressing the wild-type and mutant GLP-1 receptors. The effects of metabolic inhibitors or hypertonic medium on the rate of endocytosis were also measured (Fig. 5B). The rate of endocytosis was inhibited by 73–90% under both conditions after metabolic depletion and hypertonic treatment in cells expressing wild-type or mutant receptors.

Effect of agonist on the cellular redistribution of GLP-1 receptors

It has been reported that GLP-1 receptor recycling to the cell surface following agonist-induced endocytosis takes about 15 min and that a rapid rate of endocytosis produces a progressive decrease in the surface expression of GLP-1 receptors upon continuous exposure to the agonist.

The effect of pre-exposure to 10 nM GLP-1(7–36)amide on cell surface receptors was determined as a function of time, as shown in Fig. 5C. A rapid decrease in cell surface receptor numbers was observed in cells expressing wild-type and mutant GLP-1 receptors. In both cases, the number of cell surface receptors decreased during the first 15 min of agonist exposure, receptor numbers falling to 45–50% of the initial values. After longer times of exposure, the number of cell surface receptors decreased slowly to 36–27% in cells expressing wild-type and mutant receptors respectively.

Discussion

Here we report the results obtained following the replacement of Cys⁴³⁸ by alanine in the rat GLP-1 receptor.

The data obtained by us show that the GLP-1 receptor is palmitoylated and that substitution of Cys⁴³⁸ by alanine prevented ³H-palmitate incorporation to GLP-1 receptors. We do not have any information about the stoichiometry of GLP-1 receptor palmitoylation.

As compared with the wild-type receptor, the efficacy of mutant A⁴³⁸ receptors in stimulating adenylate cyclase activity after GLP-1(7–36)amide treatment decreased approximately threefold. A decrease in cAMP production in cells expressing mutant GLPR A⁴³⁸ receptors is consistent

Figure 5 Agonist-induced endocytosis and redistribution of GLP-1 receptors and the effect of metabolic inhibitors and hypertonic medium on endocytosis in cells expressing wild-type and mutant GLP-1 receptors. (A) The rate of [¹²⁵I]-GLP-1(7–36)amide endocytosis was measured in CHO cells expressing wild-type, GLPR A⁴³⁸, GLPR A^{431/432} and GLPR A^{431/432/438} GLP-1 receptors. Cells were incubated with 0.1 nM [¹²⁵I]-GLP-1(7–36)amide. After defined times, total and acid wash-resistant (intracellular) cell-associated radioactivity was measured. Data are represented in the form of an IN/SUR plot, means $\pm$ S.E.M. ($n=6$). IN, intracellular [¹²⁵I]-GLP-1(7–36)amide; SUR, cell surface-bound [¹²⁵I]-GLP-1(7–36)amide. (B) The effects of metabolic depletion and hypertonic medium in the internalisation process were analysed in CHO cells expressing wild-type or mutant GLP-1 receptors. The data are percentages of the rate of endocytosis and are means $\pm$ S.E.M. of three independent experiments performed in triplicate. (C) The effect of pretreatment with 10 nM GLP-1(7–36)amide on cell surface expression of GLP-1 receptors was investigated. Cells were incubated in the presence of agonist for different times, after which the cells were washed and the ligand bound to the cell surface was removed by acid washing. Finally, [¹²⁵I]-GLP-1(7–36)amide binding to cell surface receptors was measured. Results are means $\pm$ S.E.M. ($n=6$).

with the data reported for non-palmitoylated β_2 adrenergic receptors (O'Dowd *et al.* 1989) and the D1 dopamine receptor (Jensen *et al.* 1995). In our data, the differences in cAMP production are not correlated with a change in the agonist-affinity state of GLP-1 receptors after the substitution of Cys⁴³⁸ by alanine.

Replacement of Cys⁴³⁸ or substitution of Ser^{431/432} did not affect the processing of mutant receptors. Under these experimental conditions, wild-type and mutant receptors showed a similar agonist binding affinity. In contrast, the expression level of mutant A⁴³⁸ and A^{431/432} at the cell surface was higher than for wild-type GLP-1 receptors, and CHO cells transfected with mutant A^{431/432/438} expressed low levels of receptors as compared with wild-type receptors. Thus, these findings cannot be related to the effect of palmitoylation or depalmitoylation.

It has been reported that the region involved in the coupling of GLP-1 receptors to G proteins is located in the third intracellular cytoplasmic loop (Takhar *et al.* 1996) and that C-terminal tail phosphorylation is involved in the desensitisation process. Nevertheless, a receptor lacking 33 amino acids of the C-terminal tail stimulates cAMP production at the same rate as wild-type receptors (Widmann *et al.* 1996a,b). We also tested a truncated receptor lacking 27 amino acids of the C-terminal tail and this truncated form stimulated cAMP production at a similar rate to wild-type receptors, indicating that this region of the cytoplasmic tail is not necessary for the GLP-1 receptor/G protein interaction (our unpublished data).

Palmitoylation of cysteines in the carboxyl terminal tail of G-protein-coupled receptors anchors this receptor domain into the plasma membrane, forming a fourth intracellular loop. Substitution of Cys⁴³⁸ in GLP-1 receptors abolishes ³H-palmitate incorporation, which should produce a different conformational state of the carboxyl terminal tail and may affect the access of kinases to the cytoplasmic tail.

The cytoplasmic tail of GLPR also contains several phosphorylation sites reported to be necessary for the internalisation and desensitisation of GLP-1 receptors. Previous work using GLP-1 receptors revealed that PKC activation by phorbol esters produced phosphorylation of the four serine doublets in the intracytoplasmic tail: Ser^{431/432}, Ser^{441/442}, Ser^{444/445} and Ser^{451/452} (Widmann *et al.* 1996a). Agonist binding to the GLP-1 receptor also induces phosphorylation of three doublets: Ser^{441/442}, Ser^{444/445} and Ser^{451/452} (Widmann *et al.* 1997), and the receptor phosphorylation induced by homologous and heterologous desensitisation is additive (Widmann *et al.* 1996b). The substitution of Cys⁴³⁸ might regulate the access of protein kinases to such phosphorylation sites. Thus, the phosphorylation of a protein kinase A (PKA) site in the β_2 -adrenergic receptor located downstream from the palmitoylated cysteine is also dependent on the state of palmitoylation of the receptor (Moffett *et al.* 1993,

1996). It has also been reported that mutations of the two Cys residues in the A³-adenosine receptor increase phosphorylation of the receptor independently of the presence of agonist (Palmer & Stiles 2000). Palmitoylation of the vasopressin V_{1a} receptor also regulates phosphorylation, but in this case the reduction of [³H]palmitate incorporation decreased the basal level of phosphorylation of mutant receptors (Hawtin *et al.* 2001).

Since our data indicated that the rate of endocytosis was similar in wild-type and mutant receptors, and since it has been reported that phosphorylation of the three doublets Ser^{441/442}, Ser^{444/445} and Ser^{451/452} is involved in this process, we next investigated the effect of the substitution of serines 431/432 by alanines in the wild-type and GLPR A⁴³⁸ receptors. The substitution of Ser^{431/432} by alanine did not modify the potency or efficacy of these receptors in stimulating adenylate cyclase. In contrast, substitution of these residues in GLPR A⁴³⁸ receptors increased the efficacy in stimulating adenylate cyclase at concentrations higher than 1 nM GLP-1(7–36)amide.

The results obtained after the substitution of Ser^{431/432} by alanine when Cys⁴³⁸ is also absent suggest that the rate of phosphorylation of the other serine doublets in the cytoplasmic tail might also be affected. To confirm this hypothesis it would be necessary to analyse the effect of substitution of the other sites of phosphorylation involved in the desensitisation and internalisation of GLP-1 receptors. However, a synergistic action of kinases on other members of G-protein-coupled receptors, such as β_2 -adrenergic receptors (Moffett *et al.* 2001) and A₃-adenosine receptors (Palmer & Stiles 2000), has been reported previously.

The effect of the substitution of Cys⁴³⁸ by alanine on the endocytosis and recycling processes was also tested. The GLPR A⁴³⁸ receptors did not show significant differences in the internalisation rate as compared with the wild-type GLP-1 receptor and, likewise, the number of cell surface receptors after agonist exposure decreased in the same way as in cells expressing the wild-type receptors. Substitution of Ser^{431/432} by alanine in wild-type and GLPR A⁴³⁸ did not modify either the rate of endocytosis or the number of cell surface receptors after exposure to 10 nM agonist. The effect of acid washing was similar in all clones used. Our results suggest that the substitution of Ser^{431/432} or Cys⁴³⁸ by Ala in the GLP-1 receptor does not modify the capacity of these receptors to interact with the components of the vesicular transport system.

We also studied the endocytosis pathway used by these receptors, testing the effect of hypertonic medium; we found that all wild-type and mutant GLPR A⁴³⁸, GLPR A^{431/432} and GLPR A^{431/432/438} receptors were mainly internalised through coated pits.

In conclusion, the substitution of Cys⁴³⁸ in the cytoplasmic tail of GLP-1 receptors decreases the efficacy of adenylate cyclase stimulation promoted by agonist binding through these receptors. In addition, this residue is a site of

acylation of GLP-1 receptors and might regulate the state of phosphorylation of the GLP-1 receptor, which modulates the desensitisation process. Nevertheless, further studies are required to confirm the latter hypothesis.

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